The C$_4$-Dicarboxylic Acid Pathway of Photosynthesis

IDENTIFICATION OF INTERMEDIATES AND PRODUCTS AND QUANTITATIVE EVIDENCE FOR THE ROUTE OF CARBON FLOW

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1. When leaves with the C$_4$-dicarboxylic acid pathway of photosynthesis are exposed to $^{14}CO_2$ the major labelled compounds formed, in order of labelling, are dicarboxylic acids, 3-phosphoglycerate, hexose phosphates and sucrose. During the present studies several quantitatively minor intermediates were identified and their labelling behaviour is described. 2. The pattern of labelling of dihydroxy-acetone phosphate, fructose 1,6-diphosphate and ribulose di- and mono-phosphates during radiotracer pulse–chase experiments was consistent with their operation as intermediates in the pathway of carbon dioxide fixation. 3. Serine, glycine, alanine and glutamate had labelling patterns typical of products secondary to the main flow of carbon. 4. The mechanism of the transfer of label from C-4 of dicarboxylic acids to C-1 of 3-phosphoglycerate was also examined. Evidence consistent with pyruvate being derived from C-1, C-2 and C-3 of oxaloacetate, and for a relationship between ribulose 1,5-diphosphate and the acceptor for the C-4 carboxyl group, was obtained. 5. Evidence is provided that, under steady-state conditions, essentially all the label incorporated from $^{14}CO_2$ into C-1 of 3-phosphoglycerate enters via C-4 of the dicarboxylic acids. These and other studies indicated that the route via dicarboxylic acids is essentially the sole route for entry of carbon into 3-phosphoglycerate.

A scheme describing the pathway of photosynthetic CO$_2$ fixation in sugar-cane leaves, proposed on the basis of kinetic studies on $^{14}CO_2$ incorporation and the intramolecular labelling of intermediates (Hatch & Slack, 1966), has now been substantiated in many respects by enzyme studies (Slack & Hatch, 1967; Hatch & Slack, 1968, 1969; Hatch, Slack & Bull, 1969; Slack, 1969). This pathway, termed the C$_4$-dicarboxylic acid pathway, has since been shown to operate in species from several other Families besides the Gramineae, including dicotyledonous Families (Hatch, Slack & Johnson, 1967; Johnson & Hatch, 1968). Previous studies indicated that, after the carboxylation of phosphoenolpyruvate, the C-4 carboxyl group of the dicarboxylic acids so formed gives rise to C-1 of 3-phosphoglycerate, but we had no direct evidence that this was the only significant route for 3-phosphoglycerate formation. It was also apparent from these studies that several intermediates, in addition to those previously described,

remained to be identified. Presumably these intermediates exist in small pools and therefore accumulated only small proportions of the total radioactivity. Information about some of these intermediates is provided in the present paper. Evidence that the route via C$_4$ dicarboxylic acids is essentially the sole route for entry of CO$_2$ into 3-phosphoglycerate is also presented.

MATERIALS

Mature leaves of sugar cane (*Saccharum* hybrid var. Pindar), maize (*Zea mays* var. D5606A) and sorghum (*Sorghum vulgare* var. rio) were obtained from plants grown in the field or temperature-controlled glass-houses.

Ba^{14}CO$_3$, [U-^{14}C]serine, [U-^{14}C]aspartic acid and [U-^{14}C]-malic acid were obtained from The Radiochemical Centre, Amersham, Bucks. The following compounds and enzymes were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.: dihydroxyacetone, dihydroxyacetone phosphate, 2-oxoglutarate, NAD$^+$, alkaline phosphatase, malate dehydrogenase, lactate dehydrogenase and aspartate

aminotransferase. For thin-layer chromatograms silica gel (Eastman type K301-R2) was used.

CO₂-free air was prepared by passing laboratory air through NaOH, silica gel and Carbosorb. After this treatment no CO₂ was detectable with a Beckman infrared gas analyser standardized against CO₂-free N₂. Before use the air was humidified by bubbling through CO₂-free water.

METHODS

Exposure of leaves to $^{14}CO_2$

The procedure for application of $^{14}CO_2$ to leaves photosynthesizing under steady-state conditions was as described by Hatch & Slack (1966). Variations of this procedure are described in the text. Light-intensity was measured as described by Hatch & Slack (1966).

Killing of leaves and extraction of radioactive compounds

For most experiments leaves were killed in boiling 80% (v/v) ethanol and extracted as described by Hatch & Slack (1966). For the determination of radioactive bicarbonate pools the same general procedure was used except that leaves were killed at 70° in a solution containing 0.05M-KOH in 50% (v/v) ethanol. The insoluble material was re-extracted with hot aqueous 0.01M-KOH and then water and the extracts were pooled. For analysis of oxaloacetate and pyruvate leaves were killed in a 2,4-dinitrophenylhydrazine–HCl solution (Hatch & Slack, 1966).

Measurement of radioactivity

Aqueous samples were either dried on filter paper and counted with a Geiger–Müller tube at 6.5% efficiency or dried on a Whatman GF/A glass-fibre disc and counted in a Beckman L-100 scintillation counter at an efficiency of 90%. The insoluble residue containing α-glucan was counted as described by Hatch & Slack (1966).

$^{14}CO_2$ released during degradation studies was trapped on glass-fibre paper saturated with 0.1 ml. of a solution containing 0.2M-Ba(OH)₂ and 1M-NaOH, supported in the centre well of a Warburg vessel. After being dried the glass-fibre discs were counted by the liquid-scintillation procedure described above.

Identification of radioactive compounds and estimation of their radioactivity

Chromatography procedures. Paper-chromatography solvents used were as follows: A, butan-1-ol–propionic acid–water (10:4.7:6.7, by vol.) (Benson et al. 1950); B, pentan-1-ol saturated with 5M-formic acid (Aronoff, 1956); C, phenol–water (4:1, w/v) (Benson et al. 1950); D, ethyl acetate–pyridine–water (8:2:1, by vol.) (William & Bevenue, 1951); E, butan-1-ol–95% (v/v) ethanol–water (13:8:4, by vol.) (Putman, 1957); F, butyric acid–butan-1-ol–water (2:2:1, by vol.) (Aronoff, 1956); G, benzene–tetrahydrofuran (7:3, v/v) (Byrne, 1965); H, acetone–water–diethylamine (16:3:1, by vol.) (Wright & Stadtman,

1956); J, pyridine–acetic acid–water (10:7:3, by vol.) (Decker & Riffart, 1950); K, butan-1-ol–ethanol–0.5M-NH₃ (7:1:2, by vol.) (El Hawary & Thompson, 1953); L, water-saturated 3-methylbutan-1-ol (Aronoff, 1956). Either Whatman no. 1 or Whatman no. 3MM paper was used and for the chromatography of phosphate esters the paper was prewashed (Bieleski & Young, 1963). The location and estimation of the percentage of the total radioactivity in individual compounds on paper chromatograms were carried out as described by Hatch & Slack (1966).

Phosphorylated compounds. Phosphorylated compounds were separated on preparative chromatograms developed in solvent A into two groups, the monophosphates and the diphosphates. All non-phosphorylated compounds moved beyond the monophosphates. The only phosphorylated compound not in these areas was phosphoenolpyruvate, which moved with aspartic acid (Benson et al. 1950). Phosphate esters were identified by eluting the individual bands from preparative chromatograms with water, treating the eluted material with phosphatase and identifying the products by co-chromatography with marker compounds. Dihydroxyacetone was identified by its behaviour during chromatography in solvents A, C, D and F, ribulose by chromatography in solvents A, C and D, fructose by chromatography in solvents C and D, and pyruvate by chromatography in solvents A and B.

Amino acids. Serine and glycine moved with aspartate, phosphoenolpyruvate and free hexoses in preparative chromatograms developed with solvent A. Sugars were removed by chromatography in solvent D and the remaining compounds with solvent C. If glycine and serine were not completely separated with solvent C, they were eluted and chromatographed in solvent H, in which serine ran well ahead of glycine. Radioactive alanine (solvents A and C) and glutamate (solvents A, C and J) were identified by co-chromatography with their respective marker compounds.

Other compounds. The procedure used for hydroxypyruvate, glycollate and their phosphorylated derivatives involved chromatography of the leaf extracts, before and after treatment with phosphatase, in solvent B. Glycollate was determined by scanning these chromatograms after spraying the glycollate area with 0.1M-NaHCO₃ to prevent volatilization. Since hydroxypyruvate ran with glycerate in solvent B, this area of the chromatogram was eluted and these two compounds were separated in solvent A.

To test for radioactive phosphoglycolaldehyde the area in which marker phosphoglycolaldehyde runs in solvent A, which also contains aspartate and other compounds, was eluted from chromatograms. The eluted material was treated with phosphatase. After the addition of carrier glycolaldehyde the 2,4-dinitrophenylhydrazone of this compound was formed as described by Reich & Samuels (1956). The derivative was chromatographed in solvent L.

Chromatographic purification of labelled compounds for degradation studies. Malate was obtained free of other radioactive compounds by chromatography of the total aqueous extract in solvent B. Glycerate, obtained by phosphatase treatment of the total aqueous extract, was purified by chromatography in solvent A, in which it ran with malate and ribulose. The latter compounds were removed by chromatography in solvent B. Serine, glycine and aspartate were purified as described above. Oxaloacetate and pyruvate, isolated as their 2,4-dinitrophenylhydrazones,

were purified by chromatography in solvent K (Hatch & Slack, 1966).

Identification of intermediates as derivatives. Labelled dihydroxyacetone was isolated from chromatograms developed with solvent D and converted into its 2,4-dinitrophenylhydrazone (Reich & Samuels, 1956). This derivative co-chromatographed with the authentic compound on a thin-layer silica-gel plate developed in solvent G. Radioactive ribulose, isolated from chromatograms developed with solvent D, was converted into its phenylosazone (Aronoff, 1956). This derivative co-crystallized to constant specific radioactivity with authentic ribulose phenylosazone prepared by the same procedure.

Degradation of labelled compounds. Aspartic acid was converted into oxaloacetate by treatment with aspartate aminotransferase and 2-oxoglutarate. The oxaloacetate formed was decarboxylated with CuSO$_4$ (Slack & Hatch, 1967), releasing the C-4 carboxyl group as ^{14}CO$_2$. The radioactivity lost was determined by counting before and after treatment.

Malic acid was converted into aspartic acid in a system containing malate dehydrogenase, aspartate aminotransferase, glutamate and NAD$^+$, together with pyruvate + lactate dehydrogenase to regenerate NAD$^+$. Aspartate was isolated by chromatography in solvent J and then degraded as described above.

Serine was degraded with ninhydrin in the main compartment of a Warburg vessel at pH 2·5 (Aronoff, 1956) and the ^{14}CO$_2$ was trapped and counted as described above.

Glycerate (Hatch & Slack, 1966) and the 2,4-dinitrophenylhydrazone of oxaloacetate (Hatch *et al.* 1967) were degraded as previously described.

RESULTS

Labelling of intermediates and products

Phosphorylated sugars and the carboxyl acceptor. The purpose of the experiments with sorghum leaves described in Fig. 1 was twofold. The ^{12}CO$_2$ pulse–chase treatment (cf. Figs. 1a and 1c) was employed to detect and determine the pattern of flow of label through pools of quantitatively minor intermediates under steady-state conditions. In particular, intermediates lying between 3-phosphoglycerate and hexose monophosphates, as well as possible intermediates leading to the formation of the proposed carboxyl acceptor (Hatch & Slack, 1966), were sought. Treatments exactly paralleling the pulse–chase procedure, but in which the leaves were transferred to CO$_2$-free air instead of normal air, were also examined (Figs. 1b and 1d). This treatment was expected to deplete the pool of C$_4$ dicarboxylic acids and cause thereby the acceptor of the C-4 carboxyl group of the dicarboxylic acids to accumulate.

The major labelled components, previously identified in sugar cane (Hatch & Slack, 1966) and other leaves (Johnson & Hatch, 1968), are included in Figs. 1(a) and 1(b) for comparison with the results in Figs. 1(c) and 1(d). There was no appreciable loss of radioactivity from leaves during the pulses in ^{12}CO$_2$ and CO$_2$-free air. During the ^{12}CO$_2$ pulse dihydroxyacetone phosphate, fructose 1,6-diphosphate and ribulose mono- and di-phosphates gained and then lost radioactivity in a manner consistent with their operation as intermediates (Fig. 1c). By 300 sec. the amount of radioactivity in these compounds had declined to about 2% or less of the maximum quantity reached earlier in the pulse. The observation that the period of rapid labelling of hexose monophosphates preceded that for dihydroxyacetone phosphate (Figs. 1a and 1c) is discussed below.

Several differences were observed between the ^{12}CO$_2$–air pulse and the CO$_2$-free air treatment. The more rapid loss of radioactivity from the dicarboxylic acids in the CO$_2$-free air treatment presumably reflected the expected decline in the pool size of these acids (Figs. 1a and 1b). In the CO$_2$-free air treatment a higher proportion of the total radioactivity accumulated transiently in several phosphorylated intermediates (Figs. 1c and 1d). This was probably due, in large part at least, to the higher specific radioactivity of carbon entering from C$_4$ dicarboxylic acids in the CO$_2$-free air treatment owing to the absence of isotope dilution. Of more significance was the difference between the two treatments during the period after the attainment of peak radioactivity. The proportion of the total radioactivity in several phosphorylated intermediates in the ^{12}CO$_2$ pulse had declined to less than 0·1% by 150 sec. and decreased even further by 300 sec. (Fig. 1c). Although there was also a decline in CO$_2$-free air, much higher proportions of radioactivity remained in these compounds (Fig. 1d). This proportion increased rather than decreased between 150 sec. and 300 sec. At 300 sec. there was 30–40 times more radioactivity in ribulose diphosphate and fructose diphosphate in the CO$_2$-free air treatment and about 15 and 5 times more in dihydroxyacetone phosphate and ribulose monophosphate respectively (Figs. 1c and 1d).

Amino acids. The pattern of labelling for alanine and glutamate, not shown in the graphs, and for glycine and serine, suggested that these compounds were side products away from the main flow of carbon (Figs. 1c and 1d). In the CO$_2$-free air treatment a much greater proportion of the total radioactivity appeared in glycine than was observed under ^{12}CO$_2$–air.

Carboxyl transfer intermediates. Several compounds have been considered as possible acceptors or intermediates in the transfer of C-4 of dicarboxylic acids to C-1 of 3-phosphoglycerate. In the experiments described in Fig. 1 no radioactivity was detectable in either hydroxypyruvate, glycolaldehyde or their phosphorylated derivatives.
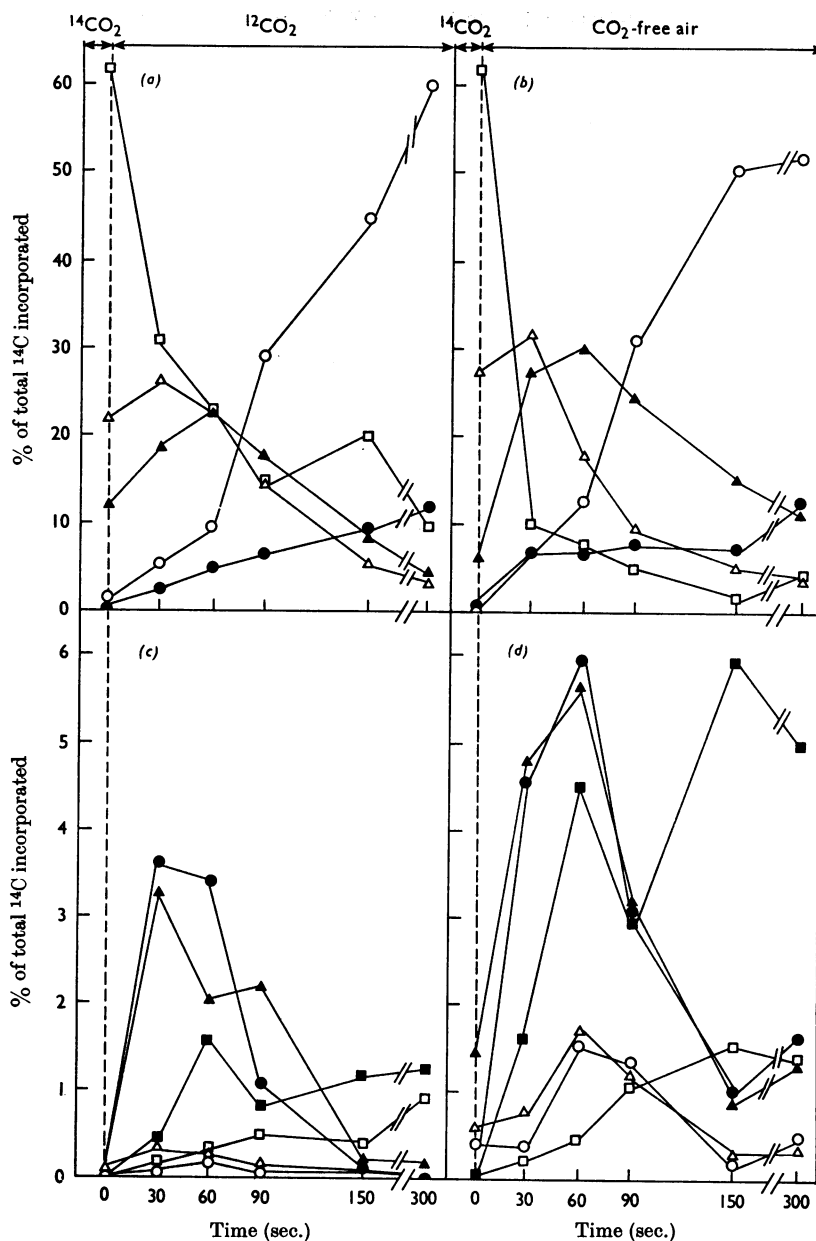
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Fig. 1. Changes in the distribution of radioactivity after transfer of sorghum-leaf segments from air containing 0·035% of $^{14}CO_2$ to air containing the same concentration of $^{12}CO_2$ (a and c) or CO_2-free air (b and d). The light-intensity was 5500 ft.-candles throughout. At intervals leaf segments were killed and analysed as described in the Methods section. For (a) and (b) the compounds were: □, malate + aspartate; △, 3-phosphoglycerate; ▲, hexose monophosphates; ○, sucrose; ●, glucan. For (c) and (d) the compounds were: ●, dihydroxyacetone phosphate; ▲, ribulose mono- and di-phosphates; ■, glycine; □, serine; △, ribulose 1,5-diphosphate; ○, fructose 1,6-diphosphate.

Hydroxypyruvate or phosphohydroxypyruvate could be possible precursors of 3-phosphoglycerate if the acceptor of the C-4 carboxyl group was a C_2 compound. Only small and variable quantities of radioactivity were detected in glycollate.

Since our failure to detect label in phospho-

hydroxypyruvate or hydroxypyruvate could have been due to the small pool size of these compounds, evidence for their existence was sought by comparing the labelling of 3-phosphoglycerate and serine. Serine could be formed directly from either phosphohydroxypyruvate or hydroxypyruvate rather than via 3-phosphoglycerate. Leaves were exposed to $^{14}CO_2$ for 60sec. and then transferred to air containing unlabelled CO_2 for a further 40sec. Although the total radioactivity in C-1 of 3-phosphoglycerate declined during the pulse in $^{12}CO_2$ as expected, the radioactivity in C-1 of serine increased 2·5-fold. From this, and observations on the changes in the proportions of the radioactivity in the individual carbon atoms of these compounds, it appeared more likely that serine was formed via 3-phosphoglycerate. The fact that more than 50% of the label in serine was located in C-1 at 60sec. was not consistent with its formation via the glycollate pathway (Rabson, Tolbert & Kearney, 1962; Hess & Tolbert, 1966).

Pyruvate, phosphoenolpyruvate and oxaloacetate. By using the 2,4-dinitrophenylhydrazine killing procedure, radioactive pyruvate was identified in sugar-cane leaves as its hydrazone. After 4sec. in $^{14}CO_2$ 0·2% of the fixed radioactivity was located in pyruvate and by 30sec. this had increased to 1·7%. This eightfold increase was accompanied by a sixfold increase in the proportion of the total radioactivity in C-1, C-2 and C-3 of oxaloacetate. In contrast 3-phosphoglycerate contained 15% of the total radioactivity at 4sec. and this increased only 2·5-fold by 30sec. Phosphoenolpyruvate apparently exists in an extremely small pool, radioactivity in this compound being undetectable after 4sec. and only barely detectable after longer periods.

Oxaloacetate was previously identified as an early labelled product of photosynthesis in plants with the C₄-dicarboxylic acid pathway (Hatch & Slack, 1966; Hatch *et al.* 1967), but its behaviour during time-course studies has not been described. When sugar-cane leaves were exposed to $^{14}CO_2$ for 10sec. and then transferred to air containing unlabelled CO_2, radioactivity was rapidly lost from oxaloacetate, as well as from malate and aspartate. After 7sec. the losses, expressed as a percentage of that present in each compound at the commencement of the pulse in air, were 55, 32 and 22% respectively. By degrading oxaloacetate it was possible to show that 64% of the radioactivity in C-4 was lost during this period.

Quantitative evidence for the path of carbon flow

Derivation of C-1 of 3-phosphoglycerate from C-4 of dicarboxylic acids. The following experiment was designed to obtain a quantitative assessment of
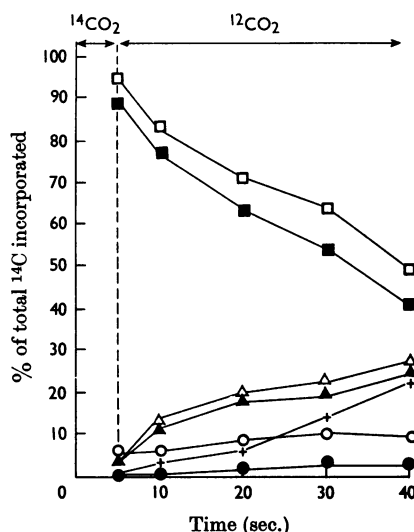

Fig. 2. Distribution of radioactivity in the compounds of maize-leaf segments exposed to air containing 0·035% of $^{14}CO_2$ for 5sec. and then transferred to air containing the same concentration of $^{12}CO_2$. The light-intensity was 2000ft.-candles throughout. Leaves were killed at intervals and compounds extracted and degraded as described in the Methods section. □, Malate+aspartate; ■, C-4 of malate+aspartate; ○, C-1, C-2 and C-3 of malate+aspartate; △, 3-phosphoglycerate; ▲, C-1 of 3-phosphoglycerate; ●, C-2 and C-3 of 3-phosphoglycerate; +, sugar phosphates.

the amount of label entering C-1 of 3-phosphoglycerate from $^{14}CO_2$ via C-4 of dicarboxylic acids. While steady-state conditions for photosynthesis were maintained maize leaves were exposed to $^{14}CO_2$ for 5sec. and some of the leaves were then transferred to air containing the same percentage of $^{12}CO_2$ for periods of up to 35sec. Other leaves were allowed to remain in $^{14}CO_2$ for periods of up to 40sec. A lower light-intensity was used in this experiment to lower the rate of movement of radioactivity, thus allowing a more accurate determination of rate curves. The transformations of radioactive material during the $^{12}CO_2$ pulse provided direct evidence that all the radioactivity lost from C-4 of dicarboxylic acids appears in C-1 of 3-phosphoglycerate (Fig. 2). By 35sec. the radioactivity in C-4 of dicarboxylic acids had fallen from 90% to 40% of the total, whereas that in 3-phosphoglycerate and C-1 thereof rose from 5% to 27% and from 5% to 25% respectively. The remainder of the radioactivity lost from the dicarboxylic acids was located in compounds that could reasonably be assumed to be derived from 3-phosphoglycerate. During the period of the $^{12}CO_2$ pulse the total radioactivity remained essentially constant.
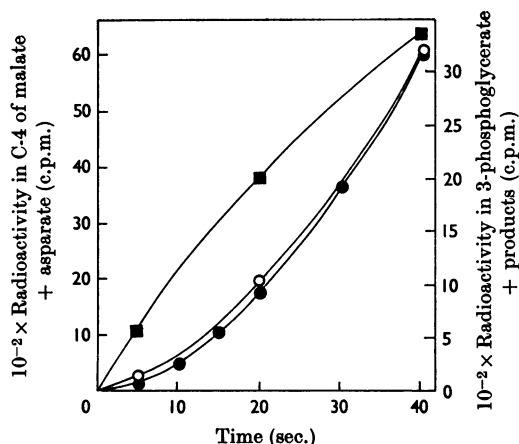
Fig. 3. Comparison of the observed radioactivity in 3-phosphoglycerate and its products and the calculated loss of radioactivity from C-4 of malate+aspartate during exposure of maize-leaf segments to air containing 0·035% of $^{14}CO_2$. These treatments were conducted simultaneously with those described in Fig. 2 but leaves were maintained in $^{14}CO_2$ for the full period of 40sec. The method of calculating the loss of ^{14}C from C-4 of malate+aspartate is described in the text. The left-hand ordinate refers to: ■, observed radioactivity in C-4 of malate+ aspartate, designated as y in the text. The right-hand ordinate refers to: ○, radioactivity in 3-phosphoglycerate and its products; ●, calculated loss of radioactivity from C-4 of malate+aspartate.

The question remaining is whether all the label entering C-1 of 3-phosphoglycerate in the presence of $^{14}CO_2$ is derived via C-4 of dicarboxylic acids. Assuming constancy of pool sizes under the prevailing steady-state conditions, the combined results of the above experiment may be used to calculate the amount of radioactivity transferred from C-4 of dicarboxylic acids to 3-phosphoglycerate during the exposure of leaves to $^{14}CO_2$. The following is the general form of the equation expressing the amount of radioactivity in C-4 of malate+aspartate, y, after a period of exposure to $^{14}CO_2$, t:

$$y = \alpha(1 - e^{-kt})$$

The constant α is equal to A/k, where A is the rate of entry of ^{14}C into C-4 of malate+aspartate and k is the rate constant for loss of ^{14}C from the same pool. The rate of loss of ^{14}C from C-4 of malate+aspartate is first-order and k was determined from the semilogarithmic plot of the data shown in Fig. 2. By using the experimentally determined values of y for the leaves remaining in $^{14}CO_2$ (Fig. 3), the value for α, and hence for A, was determined by least-squares approximation. The loss of ^{14}C from C-4 of malate + aspartate after

various times, t, in $^{14}CO_2$ was then calculated from the difference between the total entry of ^{14}C, At, and the calculated values for y after the same period. The calculated curve for loss of ^{14}C from C-4 of malate+aspartate agreed closely with the observed curve for appearance of radioactivity in 3-phosphoglycerate and its products during exposure of leaves to $^{14}CO_2$ (Fig. 3). These products include sugar phosphates, sucrose and glucan. The radioactivity in these products, together with that in 3-phosphoglycerate, would represent the radioactivity that has entered C-1 of 3-phosphoglycerate during the period in $^{14}CO_2$. If significant alternative routes were operative the radioactivity in these compounds should have exceeded that lost from C-4 of dicarboxylic acids.

Radioactive bicarbonate pools within the leaf. The operation of an alternative path for incorporation of $^{14}CO_2$ into 3-phosphoglycerate, served by a very large, and hence slowly saturating, pool of bicarbonate, may not be detected in the studies described above. However, $^{14}CO_2$ should enter such a pool at a rate equal to the ultimate potential contribution of the pathway to $^{14}CO_2$ fixation. To test for the existence of such pools maize and sugar-cane leaves were exposed to $^{14}CO_2$ for various periods and then killed in an ethanolic potassium hydroxide solution as described in the Methods section. After 20sec. between 4% and 6% of the total recovered radioactivity was present as bicarbonate and for both types of leaves this proportion declined to about 3% at 120sec. These values set an upper limit for the contribution of such an alternative process, since at least a part of this radioactivity would be likely to occur in pools not directly serving photosynthetic CO_2 fixation.

DISCUSSION

Our original proposal that the C_4-dicarboxylic acid pathway involves the operation of two cycles (Hatch & Slack, 1966) has received support from subsequent studies (Slack & Hatch, 1967; Hatch & Slack, 1968, 1969; Hatch et al. 1969; Slack, 1969). In the first cycle phosphopyruvate carboxylase catalyses the carboxylation of phosphoenolpyruvate to give oxaloacetate, which is rapidly interconverted with malate and aspartate. The C-4 of dicarboxylic acids is then transferred to an unidentified acceptor to provide C-1 of 3-phosphoglycerate, leaving pyruvate as the other product. Phosphoenolpyruvate is regenerated from pyruvate by the action of pyruvate,P_i dikinase. During the operation of the second cycle 3-phosphoglycerate is converted into the photosynthetic end products, sucrose and α-glucan, and the acceptor for the C-4 carboxyl group of dicarboxylic acids is regenerated.

As yet the transcarboxylation reaction previously proposed to account for the transfer of the C-4 carboxyl group of a dicarboxylic acid to C-1 of 3-phosphoglycerate (Hatch & Slack, 1966) has not been demonstrated.

The labelling of dihydroxyacetone phosphate and fructose 1,6-diphosphate observed during the present studies supports the view that 3-phosphoglycerate is converted into hexose phosphates by steps similar to those operative in the Calvin cycle. The presence of NADP-linked glyceraldehyde 3-phosphate dehydrogenase and alkaline fructose 1,6-diphosphatase in plants utilizing the C_4-dicarboxylic acid pathway has been reported previously (Slack & Hatch, 1967). The changes in radioactivity in individual compounds with time was also consistent with the operation of this route except that the period of rapid labelling of hexose phosphates preceded that for dihydroxyacetone phosphate. A possible explanation for the latter observation is that two pools of dihydroxyacetone phosphate are labelled, the smaller of these being labelled first and serving as a precursor of hexose phosphates.

Since leaves of plants utilizing the C_4-dicarboxylic acid pathway contain the enzymes necessary for the conversion of ribose 5-phosphate into ribulose 1,5-diphosphate (Slack & Hatch, 1967), the observation that ribulose mono- and di-phosphates were labelled was not surprising. However, the fact that these compounds were apparently turning over as rapidly as other reactants indicated that they may be intermediates in the main path of carbon flow. The possibility that ribulose 1,5-diphosphate is related to the acceptor for the C-4 carboxyl group of dicarboxylic acids received some support from its labelling behaviour after transfer of leaves from $^{14}CO_2$ to CO_2-free air. We predicted that in CO_2-free air the acceptor pool should increase after the depletion of C_4 dicarboxylic acids. In this treatment the ultimate steady amount of radioactivity in ribulose 1,5-diphosphate was about 40 times that obtained in the $^{12}CO_2$ pulse. The reason why the higher quantities of radioactivity in ribulose 1,5-diphosphate in the CO_2-free air treatment was accompanied by comparably higher amounts of radioactivity in dihydroxyacetone phosphate and fructose 1,6-diphosphate is not apparent. A possible explanation may be that glycolaldehyde phosphate, derived from C-4 and C-5 of ribulose 1,5-diphosphate, is the carboxyl acceptor. If such a cleavage of ribulose 1,5-diphosphate were catalysed by an aldolase-type reaction the other product would be dihydroxyacetone phosphate. This may be the source of the larger of the two pools of dihydroxyacetone phosphate proposed on the basis of the labelling studies described in Fig. 1. Dihydroxyacetone phosphate so formed could be the source

of a second pool of fructose 1,6-diphosphate. The fact that C-4 and C-5 are probably the last-labelled carbon atoms of ribulose 1,5-diphosphate (Bassham, 1964) may explain our failure to detect glycolaldehyde phosphate.

An explanation of the transient changes in radioactivity in ribulose 1,5-diphosphate, or indeed other compounds, in CO_2-free air would be speculative because of the non-steady-state conditions prevailing. The position is further complicated by the fact that depletion of the dicarboxylic acid pool is not instantaneous, that the specific radioactivity of the carbon leaving this pool would be transiently higher than that in the $^{12}CO_2$ pulse and that there is likely to be a small continuing production of dicarboxylic acids throughout due to fixation of internally derived CO_2. Presumably at least part of the decline in radioactivity in intermediates was due to a fall in pool size rather than replacement of labelled carbon with unlabelled carbon as would occur in a $^{12}CO_2$ pulse. We have placed special significance on the higher amounts of radioactivity that ultimately accumulated in ribulose 1,5-diphosphate, fructose 1,6-diphosphate and dihydroxyacetone phosphate in the CO_2-free air treatment compared with that in the $^{12}CO_2$ pulse. It should be noted that the difference in radioactivity in 3-phosphoglycerate, hexose monophosphates and ribulose monophosphate during the later times of the two treatments was small or negligible.

We were unable to obtain convincing evidence that a C_2 compound is the acceptor of the C-4 carboxyl group of dicarboxylic acids. The accumulation of greater quantities of radioactivity in glycine in the CO_2-free air treatment may have reflected the presence of an increased pool of a C_2 compound. Traces of glycollic acid were also detected. However, no label was observed either in glycolaldehyde phosphate, or in C_3 compounds such as phosphohydroxypyruvate, which may serve as intermediates between the transcarboxylation reaction and 3-phosphoglycerate.

The previous conclusion (Hatch & Slack, 1966) that label entering 3-phosphoglycerate is derived largely via the dicarboxylic acids was deduced indirectly from the observations that: (a) the initial rate of labelling of dicarboxylic acids was comparable with the rate of total $^{14}CO_2$ fixation, and that the latter rate was essentially linear; (b) the radioactivity rapidly lost from dicarboxylic acids, after the transfer of labelled leaves to air containing $^{12}CO_2$, appeared in 3-phosphoglycerate and its products. The pulse–chase data in Fig. 2 confirm the previous proposal (Hatch & Slack, 1966) that radioactivity is transferred from C-4 of dicarboxylic acids and appears exclusively in C-1 of 3-phosphoglycerate. Such findings do not

exclude the possibility of alternative routes mediating the incorporation of $^{14}CO_2$ into 3-phosphoglycerate. However, the present studies provide evidence that the contribution of such alternative routes must be small or insignificant. The calculated rate of loss of radioactivity from C-4 of dicarboxylic acids during exposure of leaves to $^{14}CO_2$ agreed closely with the observed rate of appearance of radioactivity in 3-phosphoglycerate and its products. Direct analysis of the radioactivity in leaf bicarbonate pools ruled out the possibility of an alternative route for labelling of 3-phosphoglycerate served by a large and hence slowly saturating bicarbonate pool.

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